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Distinctive AgNOR Patterns of Myeloid and Lymphoid Blasts in Acute Leukemia

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Bone-marrow aspiration smears of 38 cases of acute leukemia (19 acute lymphoblastic leukemia, 19 acute nonlymphocytic leukemia) were stained for argyrophilic nucleolar organiser regions (AgNOR). AgNOR were assessed numerically and morphologically. There were highly significant differences in AgNOR morphology between acute lymphoblastic leukemia and acute nonlymphocytic leukemia ANLL: lymphoblast AgNORs were usually small (<3 μm) "dots/chips," whereas myeloblasts showed larger "blebs," or a combination of the two types (complex structures). AgNOR number was significantly less in acute lymphoblastic leukemia. In acute nonlymphocytic leukemia cases, AgNOR number also showed direct correlation with Ki67 reactivity of leukemic blasts. Am. J. Hematol. 61:149–152, 1999. © 1999 Wiley-Liss, Inc.

Key words: nucleolus organizer region; leukemia; lymphocytic; nonlymphocytic

INTRODUCTION

Nucleolar organiser regions (NORs) are segments of DNA which transcribe ribosomal RNA. The NORs contain proteins that are argyrophilic (AgNOR) and are demonstrated by a simple silver staining technique [1,2]. The number and size of AgNOR have been reported to correlate with the level of rRNA transcription and number of proliferating cells [3,4].

There are few published reports [5–8] about AgNOR number and configurations in leukemic blasts, which is the basis for the present study.

MATERIALS AND METHODS

Marrow aspirates were obtained from 38 patients: acute nonlymphocytic leukemia (ANLL, 19 cases) and acute lymphoblastic leukemia (ALL, 19 cases). These cases were diagnosed with standard morphological, cytochemical and immunophenotypic (APAAP immunostain) criteria. Monoclonal antibodies used were: CD13, CD33 and CD14 (ANLL-markers); CD2 and CD3 (T-lineage); and CD19, CD20, CD10 and CD22 (B-lineage).

Staining for AgNOR

The staining method has been described previously [5]. Air dried smears fixed for 5 min in methanol, were stained for 35 min with a 2:1 mixture of 50% silver nitrate solution and 2% gelatin dissolved in 1% aqueous formic acid. Nuclei stained yellowish-brown within which AgNOR stained dark-brown and were easily identified (Fig. 1). A minimum of 200 cells were counted in each case to determine the mean number of AgNORs per cell. Cells were also grouped according to number of AgNOR structures in their nuclei (Table I).

Morphometry was done with the use of an optical micrometer disc fitted to the eyepiece of a light microscope at a total magnification of ×1000. AgNOR morphology was evaluated according to a simplified version of the method of Nikicicz and Norback [5] with minor changes in terminology as follows:

AgNOR Structures

Type I: < 3μm size, dots/chips (irregular dots). Type II: ≥3μm, blebs. Blast cells were classified into five categories (configurations) according to the type and number of AgNOR (Table II). For every case, the per-

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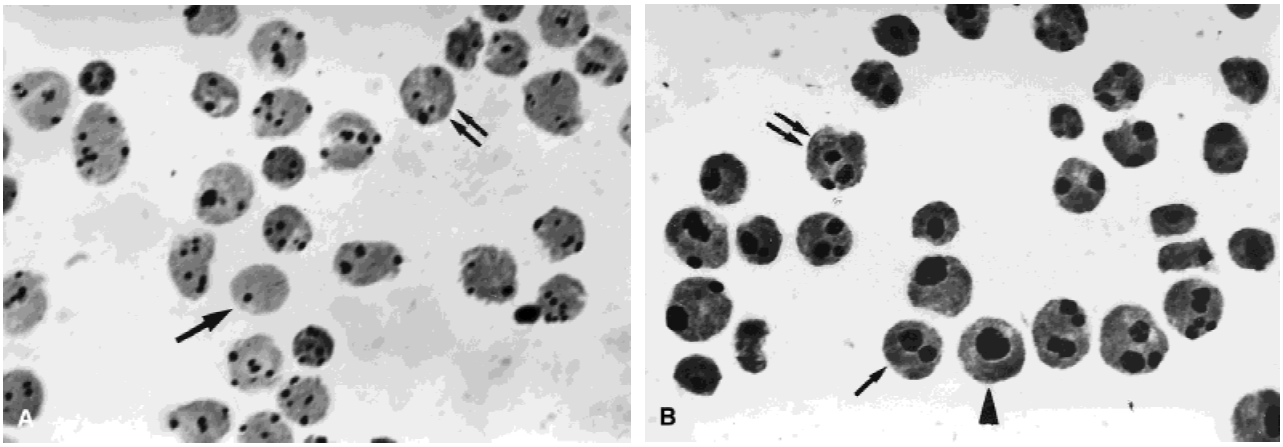

Fig. 1. AgNOR structures and configurations in acute leukemia. (a) Lymphoblasts in ALL: majority show dots/chips (type I structures) which are single (arrow) or multiple (double arrow) (b) Myeloblasts in ANLL: majority show large blebs (type II structures) which are single (arrow-head) or multiple (arrow) or complex (combined with type I structures, double arrow). AgNOR stain, original magnification ×1000.

TABLE I. AgNOR Counts and Ki67 Reactivity in Acute Leukemia

Cases (no.)	AgNOR/Cell[a] 0–1	2–3	≥4	Mean AgNOR/cell (range)	Ki67 reactivity mean (range)
ALL	17.5[b]	41.3	41.2[b]	3.2[c]	2.6
(19)	±9.9	±10.1	±15.5	(1.9–4.6)	(<1–29)
AML	7.3	39.0	54.0	3.7	4.6
(19)	±3.7	±9.9	±11.9	(3.2–4.5)	(<1–22)

[a]Numbers represent percentages of cells showing corresponding AgNOR numbers.
[b]$P < 0.001$.
[c]$P < 0.05$.

TABLE II. AgNOR Configurations* in Acute Leukemia

Cases (no.)	Type I (dots/chips) 1	>1	Total	Type II (blebs) 1	>1	Total	Complex
ALL	12.8	80.8	93.6[a]	1.2		1.2[b]	5.2[a]
(19)	±12.9	±12.8	±4.5	±1.2	0	±1.2	±4.2
AML	5.7	48.6	54.3	9.6	1.5	11.2	34.5
(19)	±4.4	±14.3	±14.6	±9.2	±1.4	±9.3	±13.4

*Numbers represent % cells.
[a]$P < 0.001$.
[b]$P < 0.01$.

centage of blasts showing each of these configurations were enumerated.

Simple configurations. Cells with structures of same type: single or multiple type I or type II structures. **Complex configurations.** Presence of both type I and type II structures in the same cell.

Ki67 Staining

Marrow smears were immunostained with primary monoclonal antibody Ki67, using the APAAP technique.

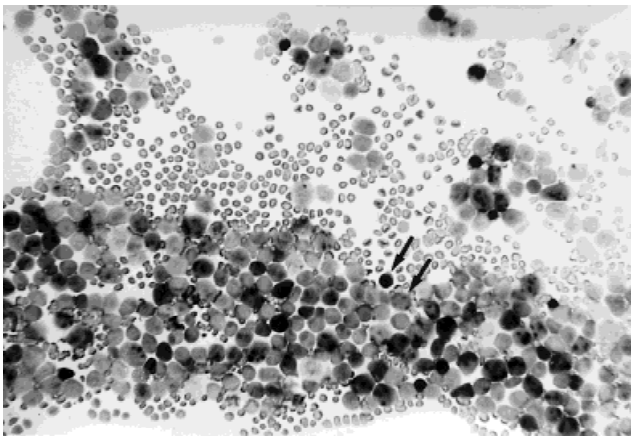

Fig. 2. Ki67-immunostained cells. Positive cells show diffuse or localised nuclear stain (arrows). APAAP stain, original magnification ×250.

The Ki67+ cells were detected by nuclear positivity (Fig. 2), and percentage positivity was enumerated after counting at least 200 cells.

Statistical Analysis

Data related to AgNOR number and configurations in ANLL and ALL were compared using Students *t*-test. Correlation coefficient (r) was calculated to analyse the relation between numbers of AgNOR and the percentage of Ki67 positive cells.

RESULTS

The ALL cases were classified as follows: L1 (11), L2 (7), and L3 (1). Immunophenotyping showed these were B-lineage (16) and T-lineage (3). ANLL cases were M1(6), M2(9), M4(3), and M6(1).

AgNOR Counts

Table I shows mean numbers of AgNORs in ALL and ANLL. Mean AgNOR in ANLL was significantly higher than in ALL ($P < 0.05$). In ALL, a higher number of cells showing 0–1 AgNOR structures were seen, whereas greater number of myeloid blasts showed ≥4 AgNOR. These were highly significant differences ($P < 0.001$). The differences between morphological subtypes of ALL (L1 v L2) and ANLL (M1 v M2) were insignificant. Myelomonocytic leukemia (M4) showed high AgNOR counts (mean 3.99/cell) and in the ALL group, B-lineage cases showed higher mean AgNOR compared to T-ALL (3.24 v 2.79) but few cases of M4 and T-ALL precluded statistical analysis.

AgNOR Structures and Configurations

Type I structures were seen more frequently in ALL compared to ANLL ($P < 0.001$). Type II and complex structures were more frequently seen in ANLL and these differences were also highly significant ($P < 0.01$ and $P < 0.001$ respectively). In M1, higher number of blasts with complex configurations and fewer with type I structures compared to M2 were seen. Differences between L1 and L2 were not significant.

Ki67 Expression

The mean number of Ki67+ cells were higher in the ANLL group compared to ALL; however, there was broad overlap of values and the difference between the two groups was not statistically significant. No difference was observed between FAB classes (L1 v L2 and M1 v M2).

Correlation between AgNOR number per cell and Ki67 positivity

There was significant overall correlation when data of ALL and ANLL were combined ($r = 0.42$, $P = 0.009$). However, this was found to be mainly due to highly significant correlation in the subgroup of ANLL cases ($r = 0.64$, $P = 0.003$). There was no correlation within the ALL group.

DISCUSSION

AgNOR may be evaluated in different ways. Simple numerical counts of AgNOR spots has been used commonly. Klobusicka observed significantly higher AgNOR count in ANLL marrow (though not in peripheral blood) as compared to ALL (7), but Nakamura et al. found no significant difference though higher counts were observed in ANLL [6]. Our study observed statistically significant higher number of AgNOR/cell in ANLL.

Although previous studies have shown that AgNOR counts correlate with other markers of cell proliferation such as BrDU uptake or Ki67 reactivity, these were mostly related to nonhemopoietic tumors [3,4,8]. Of two published studies, which attempted to correlate leukemic S-phase cells with AgNOR counts, one failed to show any correlation with BrDU labelling [6] while the other showed significant correlation using flow cytometry [7]. We used Ki67 labelling which measures the growth fraction of cells, and found significant correlation with AgNOR count in ANLL cases but not in ALL.

Numerical counts do not reflect variations of AgNOR morphology. Studies have demonstrated the importance of evaluating AgNOR morphology [9] or quantification of the AgNOR-stained areas [3]. AgNOR morphology may be evaluated according to the method of Crocker et al [10], by counting dots and clusters (clusters of dots within a matrix) separately. The method used in the present study is a simplified form of Nikicicz and Norback's method [5], based on classifying AgNOR as small structures (<3μm, type I) and larger structures (≥3μm, type II). These methods of evaluation may be interrelated because it has been observed that blebs consist of coalesced dots [5].

In normal marrow, myeloblasts usually show multiple AgNORs (mean 3.5/cell) and complex configurations [5]. AgNOR number and size decrease with myeloid differentiation. Complex configurations also characterised leukemic myeloblasts in our study.

There were significant differences in AgNOR morphology between ANLL and ALL. Approximately 45% of nonlymphoid blasts showed large AgNOR structures (blebs) which were usually in complex configurations. More than 90% lymphoblasts, on the other hand, showed single or multiple small dots.

Normal marrow cells of different lineages may be identified by characteristics of AgNOR morphology and number [5]. Similarly, AgNOR patterns in leukemic blasts of myeloid and lymphoid lineages are quite distinctive. Numerical counts and morphological assessment of AgNOR may be an additional parameter to study proliferation and phenotype of leukemic cells. The relation of these features to subtypes of ALL and ANLL are now being investigated.

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